VOL. 54 NO. 12, DEC. 2001 THE JOURNAL OF ANTIBIOTICS pp. 1060 — 1065

CJ-15,801, a Novel Antibiotic from a Fungus, *Seimatosporium* sp.

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(Received for publication August 20, 2001)

A novel antibiotic, CJ-15,801 (**I**), was isolated from the fermentation broth of a fungus, *Seimatosporium* sp. CL28611. The structure was determined to be a pantothenic acid analog having an α,β-unsaturated carboxylic acid moiety by spectroscopic analyses. The compound inhibits the growth of multi-drug resistant (MDR) *Staphylococcus aureus* strains with MIC ranging from 6.25 to 50 μg/ml.

During the past decades much effort has been devoted worldwide to limiting the spread of methicillin-resistant *Staphylococcus aureus* (MRSA). In addition to MRSA, emergence of almost untreatable vancomycin-resistant enterococci and the threat of transfer of glycopeptide resistance to *Staphylococcus aureus*[1,2] have led to a new and unexpected public health problem in hospitals and the community. Accordingly, the discovery and development of new anti-multi-drug resistant bacterial agents have become urgent.

In the course of our screening for discovery of new antibiotics, a fungus, *Seimatosporium* sp. CL28611 was found to produce a novel antibiotic, CJ-15,801 (**I**), which shows antibacterial activity against Gram-positive MDR bacteria. In this paper, we report the taxonomy of the producing organism and the fermentation, isolation, structure elucidation and biological activities of **I**.

Results and Discussion

Taxonomy

The cultural characteristics of strain CL28611 are shown in Table 1. On potato dextrose agar the conidiomata were acervular, separate, brown to black and measured 360 to 760 μm diameter. Conidiophores were septate, branched, filiform and measured 16~24×1.2~1.8 μm. The conidiogeneous cells were hyaline, holoblastic, annellidic, integrated, indeterminate and measured 12~20×1~1.6 μm. The conidia were fusiform, straight, or curved, smooth, 3-septate, not constricted at the septa and measured 13~19×2~3 μm; the medium cells were pale gray and 9~13 μm long; the apical cells hyaline or pale gray with an unbranched appendage 9~14 μm long; the basal cells were hyaline or pale gray, truncate at the base, with an unbranched, eccentric appendage 9~12 μm long. Conidiomata on oatmeal agar and cornmeal agar were smaller, measuring 280 to 580 μm diameter and 200 to 380 μm diameter, respectively.

The strain is characterized by the light ochraceous-

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Abbreviation: Amp, ampicillin; cef, cefotaxime; cip, ciprofloxacin; chl, chloramphenicol; ery, erythromycin; gent, gentamicin; kan, kanamycin; meth, methicillin; MLS_B, Macrolide, lincosamide, streptogramin B; pen, penicillin; str, streptomycin; tet, tetracycline; trm, trimethoprim; van, vancomycin.

Table 1. Cultural characteristics of strain CL28611.

	Colony Diameter	Colony Texture	Colony Surface	Colony Reverse	Soluble Pigment
Malt Extract Agar	6.0 cm	Lowly floccose to funiculose, smooth	Onion-skin pink, pecan brown (XXVIII) to light pinkish cinnamon (XXIX)	Ochraceous-salmon	None
Cornmeal Agar	6.4 cm	Slightly fluffy, thin, smooth	Colorless to light ochraceous-salmon (XV)	Same as surface	None
Potato Dextrose Agar	6.7 cm	Moderately raised funiculose, smooth	Warm buff, ochraceous-tawny, cinnamon-brown (XV), grayish olive to dark grayish olive (XLVI)	Dark olive-gray (LI) to black	None or maize yellow (IV)
Oatmeal Agar	6.7 cm	Lowly floccose or funiculose, smooth	Cinnamon to tawny-olive (XXIX)	Light pinkish cinnamon to tawny-olive	None
Cherry Juice Agar	7.3 cm	Lowly to moderately raised, funiculose, smooth	Tawny, mars brown (XV) to chestnut brown (XIV) with warm buff to antimony yellow (XV) aerial mycelium	Ochraceous salmon, mars brown (XV) to dark grayish olive (XLVI)	None

salmon, light pinkish cinnamon, pinkish cinnamon to brown colony surface; the light ochraceous-salmon, pinkish cinnamon, ochraceous salmon, olive-gray to black colony reverse and the 3-septate, fusiform conidia with an apical appendage and a basal appendage. The conidiomata are acervular. Conidiogenesis is holoblastic and annelliddic. The cells of the conidia are either concolored or pale gray in three center cells with end cells being hyaline. The basal appendage is exogenous. These features fit into the descriptions of the genus *Seimatosporium*, as defined by SUTTON[3].

The culture CL28611 resembles *S. discosioides* (Ell. & Ev.) Shoemaker[4] in the length, shape and number of septation of conidia but differs in the narrower conidia and the slightly longer appendages. Most works in this group of the Coelomycetes have been based on dried herbarium material; when members of the group are grown in culture, they are shown to be more variable than the field collection. Thus, the culture CL28611 is identified as a new strain of the genus *Seimatosporium* Corda and designated *Seimatosporium* sp.

Isolation

The fermentation broth (900 ml) treated with the same volume of EtOH at 4°C overnight was filtered and then concentrated to an aqueous solution. The resulting solution (500 ml) was extracted with *n*-BuOH (500 ml) and the organic layer was evaporated to dryness (6.1 g). A portion (200 mg) of the extract dissolved in MeOH (2 ml) was applied onto preparative HPLC on an ODS column (YMC-pack ODS AM-343, 20×250 mm+20×50 mm, YMC Co. Ltd.) with linear gradient system of MeCN-H_2O (1:9 to 1:0 for 50 minutes) at a flow rate of 8 ml/minute. The active eluate was re-applied onto the same column with linear gradient system of MeCN-0.05% TFA in H_2O (10:10 for first 30 minutes and then 10:10 to 11:9 for 30 minutes) at a flow rate of 10 ml/minute. The eluted peak was collected and concentrated to give 9.7 mg of **I** as white amorphous powder.

Physico-chemical Properties

The physico-chemical properties of **I** are summarized in Table 2. It is soluble in MeOH and DMSO. The UV spectrum (MeOH) showed a maximum absorbance at 263 nm (ε 15,000). The IR spectrum (KBr) showed absorption at 1681 and 1633 cm^{-1}, suggesting the presence of two carbonyl groups.

Table 2. Physico-chemical properties of CJ-15,801 (**I**).

Appearence	White amorphous powder
Molecular weight	217
Molecular formula	$C_9H_{15}NO_5$
HRFAB-MS (*m/z*)	found 218.1034 $[M+H]^+$ calcd. 218.1029
$[\alpha]_D^{24}$	+47.7 (*c* 0.44, MeOH)
UV λ_{max}^{MeOH} nm (ε)	263 (15,000)
IR ν_{max}^{KBr} cm^{-1}	3320, 1681, 1633, 1500, 1394, 1362, 1207, 1156, 1070, 1041, 903, 854

Table 3. ^1H and ^{13}C NMR chemical shifts of CJ-15,801 (**I**).

No.	δ_C		δ_H
1	172.4	s	
2	104.0	d	5.69 (1H, d, J = 14.3 Hz)
3	139.8	d	7.94 (1H, d, J = 14.3 Hz)
1'	175.8	s	
2'	77.9	d	4.02 (1H, s)
3'	41.6	s	
4'	70.6	t	3.48 (1H, J = 11.1 Hz)
			3.37 (1H, J = 11.1 Hz)
3'-Me	22.3	q	0.93 (3H, s)
3'-Me	21.3	q	0.93 (3H, s)

Chemical shifts are referred to CD_3OD at 49.8 ppm for ^{13}C and at 3.30 ppm for ^1H.

Structural Elucidation

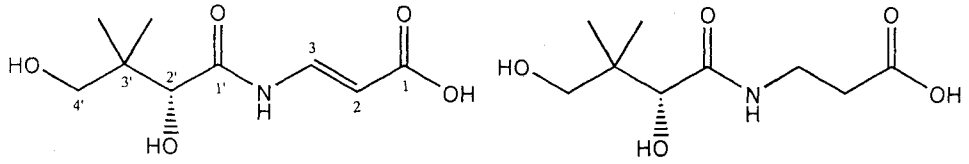
The molecular formula of **I** was determined to be $C_9H_{15}NO_5$ on the basis of HRFAB-MS (*m/z* found 218.1034, calcd 218.1029 for $C_9H_{16}NO_5$ $[M+H]^+$). The ^1H and ^{13}C NMR spectra (CD_3OD) showed 11 protons and 9 carbons, supporting the molecular formula (Table 3). The carbons were classified into two $-CH_3$, one $-O-CH_2-$, one $-O-CH-$, one quaternary, two $=CH-$ and two carbonyl carbons by analysis of the DEPT spectra. The connectivity of proton and carbon atoms was assigned by the ^{13}C-^1H COSY spectrum as shown in Table 3.

As shown in Fig. 2, the structure of **I** was elucidated from the results of ^1H-^1H COSY and COLOC experiments. In the COLOC experiment, two singlet equivalent methyl groups, 3'-Me (δ 0.93), were coupled to oxy-methine carbon (C-2', δ 77.9), quaternary carbon (C-3', δ 41.6) and oxy-methylene carbon (C-4', δ 70.6). Moreover, the long-range couplings were observed from 2'-H (δ 4.02) to C-1' (δ 175.8) and C-3' and from 4'-H (δ 3.48, 3.37) to C-3'. These data revealed the presence of 2,4-dihydroxy-3,3-dimethyl-oxo-butyl group (from C-1' to C-4') in **I**. This structure was also supported by comparing the chemical shifts of ^1H and ^{13}C NMR with those of the structurally related compound, pantothenic acid[5]. The presence of the α,β-unsaturated carboxylic acid, which was thought to be dehydrogenated pantothenic acid, was shown by the ^1H-^1H COSY and COLOC experiments as follows. The ^1H-^1H COSY revealed a proton sequence, $-C_2H=C_3H-$. In the COLOC experiment, the higher field olefinic proton, 2-H, had the long-range couplings with C-1 (δ 172.4) and the lower field olefinic proton, 3-H, coupled with C-1 and C-1'. These data indicated that C-1 was attached to C-2 and C-1' was also bonded to C-3 through a nitrogen atom, where the connection of C-3 and C-1' by a nitrogen was determined by the nitrogenated feature of the ^{13}C NMR chemical shift at C-3 (δ 139.8). The geometry of the olefin was determined as *E* from the coupling constant (J=14.3 Hz) between 2-H (δ 5.69) and 3-H (δ 7.94). The configuration at C-2' was elucidated to be *R* by comparison of the $[\alpha]_D$ value between **I** [+47.7 (MeOH)] and pantothenic acid [*R*-form=+37.5 (H_2O)[6], *S*-form=−56.3 (MeOH)[7]]. Therefore, the structure of CJ-15,801 was elucidated as shown in Fig. 1.

Biological Activities

Compound **I** showed antibacterial activity only against MDR *Staphylococcus aureus* strains with MIC ranging from 6.25 to 50 μg/ml (Table 4). On the other hand, (*R*)-pantothenic acid that structurally resembles **I** has no antibacterial activity, but rather it is a growth-promoting factor for rat, microorganisms and plants[8~10]. It is worthwhile to investigate the mode of action for **I**.

Fig. 1. Structures of CJ-15,801 (**I**) and (*R*)-pantothenic acid.



I (*R*)-Pantothenic acid

Fig. 2. ^{1}H-^{1}H COSY and COLOC experiments of CJ-15,801 (**I**).

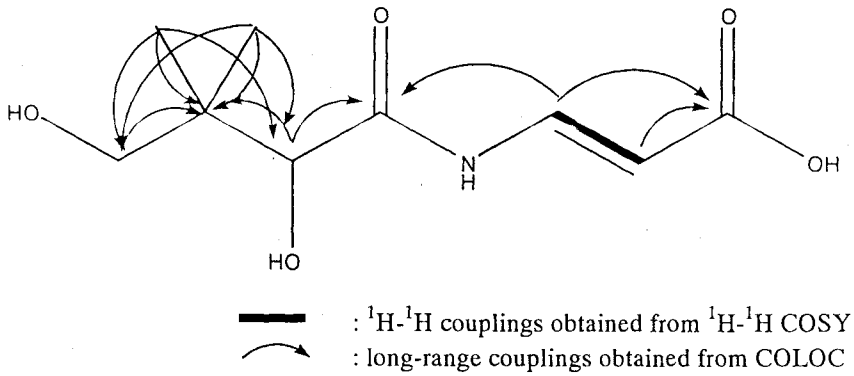


Table 4. Antibacterial activities of CJ-15,801 (**I**).

Microorganism	MIC (μg/ml)			
	CJ-15,801 (**I**)	Erythromycin	Azithromycin	Vancomycin
Staphylococcus aureus 01A1095	25	>100	>100	0.78
S. aureus 01A1105	6.25	>100	>100	1.56
S. aureus 01A1120	50	>100	>100	0.39
S. haemolyticus 01E1006	>100	100	>100	0.78
Streptococcus agalactiae 02B1023	>100	>100	>100	0.39
S. pyogenes 02C1068	>100	>100	>100	0.39
S. pyogenes 02C1079	>100	>100	>100	0.2
S. pneumoniae 02J1046	>100	>100	>100	0.39
S. pneumoniae 02J1095	>100	>100	>100	0.31
Enterococcus faecalis 03A1069	>100	>100	>100	50
Haemophilus influenzae 54A0085	>100	3.12	<=0.2	Not tested
H. influenzae 54A0131	>100	3.12	<=0.2	>100
Moraxella catarrhalis 87A1055	>100	0.78	<=0.2	50
Escherichia coli 51A0266	>100	100	1.56	>100

Experimental

General

Spectral and physico-chemical data were obtained by the following instruments: UV, JASCO Ubest-30; IR, Shimadzu IR-470; NMR, JEOL JNM-GX270 equipped with an LSI-11/73 host computer, TH-5 tunable probe and version 1.6 software; LRFAB- and HRFAB-MS, JEOL JMS-700 (MStation); optical rotations, JASCO DIP-370 with a 5 cm cell. All NMR spectra were measured in CD_3OD unless otherwise indicated and peak positions are expressed in parts per million (ppm) based on the internal standard of the CD_3OD peak at 3.30 ppm for ^1H NMR and 49.8 ppm for ^{13}C NMR. All FAB-MS spectra were measured using glycerol-matrix.

Producing Microorganism

The strain CL28611 was isolated from a soil sample collected in Handa, Aichi Prefecture, Japan. It was block or smear inoculated from a block or a spore suspension of a malt extract agar slant onto plates of identification media and the plates were incubated at 25°C in the dark for a week and then under alternate black light and darkness 12 hours each for two weeks. The results were read at two weeks for cultural characteristics and at 3 weeks for the temperature study. The colors were determined by comparisons with color chips from RIDGWAY[11]. Identification media used are cornmeal agar[12], malt extract agar[13], potato dextrose agar (peeled potato 100 g, dextrose 10 g, agar 20 g, coconut milk 50 ml, tap water 950 ml), oatmeal agar (oatmeal 30 g, agar 15 g, distilled water 1 liter) and cherry juice 200 ml, calcium carbonate 3 g, agar 15 g, tap water 800 ml). Malt extract agar was used for temperature study.

Fermentation

The culture CL28611 was maintained on a potato dextrose agar slant (Difco). A vegetative cell suspension from the slant culture was inoculated into a 500-ml flask containing 100 ml of a seed medium (potato dextrose broth 2.4%, yeast extract 0.5% and agar 0.1%). The flask was shaken at 26°C for 4 days on a rotary shaker with 7-cm throw at 210 rpm to obtain a seed culture.

The seed culture (5 ml) was used to inoculate into nine 500-ml flasks containing 100 ml of a production medium (glucose 1%, glycerol 6.6%, NZ Amine Type A 0.5%, ammonium sulfate 0.2%, defatted soybean meal 0.5%, tomato paste 0.5% and sodium citrate 0.2% and adjusted to pH 7.0) and 30 g buckwheat. Static fermentation was carried out at 26°C for 18 days.

HPLC Analysis

Analytical HPLC was performed on an ODS column (YMC-pack ODS AM-310-3, 4.6×50 mm, YMC Co. Ltd.) with MeCN - 0.05% TFA in H_2O (1 : 1) at a flow rate of 0.9 ml/minute. Under these conditions, **I** was eluted at the retention time of 4.5 minutes.

Test Strains

S. aureus 01A1105 (cef^r, $gent^r$, $meth^r$, $MLS_B{}^r$, pen^r, tet^r, cip^r, van^s, where r and s meant a resistant and sensitive strain, respectively) and *S. aureus* 01A1095 (amp^r, cef^r, $gent^r$, imipenem^s, $MLS_B{}^r$, tet^r, van^s) are MDR clinical strains. *S. aureus* 01A1120 exhibits a constitutive MLS_B-resistant phenotype due to the presence of a plasmid pE194 containing *ermC*. *Staphylococcus haemolyticus* 01E1006 is resistant to 14- and 15-membered macrolides, streptogramin B and trm. *Streptococcus pyogenes* 02C1068 is $MLS_B{}^r$, kan^r and str^r. *Str. pyogenes* 02C1079 is also $MLS_B{}^r$. *Streptococcus agalactiae* 02B1023 and *Streptococcus pneumoniae* (serotype 6) 02J1046 are $MLS_B{}^r$ and tet^r. *S. pneumoniae* 02J1095 (serotype 3) is $MLS_B{}^r$, pen^r, tet^r and trm^r. *Enterococcus faecalis* 03A1069 is also an MDR clinical strain [cef^r, ery^r, $gent^r$, chl^r, kan^r, tet^s and van^r], confirmed to have *ermB* gene. *Haemophilus influenzae* 54A0085 and 54A0131 are both type B and trm^r isolates; the former is pen-sensitive whereas the latter is pen-resistant. *Moraxella catarrhalis* 87A1055 is pen-resistant and shows intermediate susceptibility to ery. *Escherichia coli* 51A0266 is a generally susceptible strain.

Preparation of Inoculum and MIC determinations

Preparation of the inoculum, antibacterial assay and microtiter-based MIC determinations were done according to the National Committee for Clinical Laboratory Standards[14]. Erythromycin, azithromycin and vancomycin were used as standard antibiotics.

Acknowledgments

We would like to thank Dr. ARTHUR E. GIRARD and his colleagues for their measurements of biological activities.

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